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Analysis of Immune Responses of Different Hosts to *Babesia divergens* Isolates from Different Geographic Areas and Capacity of Culture-Derived Exoantigens To Induce Efficient Cross-Protection

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The immunoprecipitation of [^{35}S]methionine-radiolabelled antigens from different *Babesia divergens* isolates by using bovine, gerbil, and human immune sera has shown that many *B. divergens* proteins contain epitopes shared between isolates. The cross-protective capacity of culture-derived soluble immunogens from the *B. divergens* Rouen 1987 isolate was tested against different *B. divergens* isolates. Results showed complete protection against the 7107b French isolate and substantial protection against the Weybridge 8843 English isolate (80% protection) and the Munich 87 German isolate (60% protection). In order to explain these vaccination results and to assess both the common and variable antigenicity of *B. divergens*, the antigenic patterns of the challenge isolates (Rouen 1987, 7107b, Weybridge 8843, and Munich 87) were compared by immunoprecipitation, using gerbil antisera raised against the Rouen 1987 vaccine isolate. Differences in the antigenic patterns and in the cross-protection of gerbils in these heterologous challenges were examined by studying the virulence and the antigenic status of each isolate.

In Europe, the intraerythrocytic protozoan parasite *Babesia divergens*, transmitted by the three-host cattle tick *Ixodes ricinus*, is the most pathogenic *Babesia* species affecting cattle (12). Moreover, several cases of *B. divergens* infection have also been described in splenectomized human patients (7, 10). Recently, we have developed a new method for the long-term in vitro cultivation of *B. divergens* and proposed a culture-derived vaccine containing *B. divergens* exoantigens as a means to control bovine babesiosis (8). The protective capacity of these culture-derived exoantigens with the adjuvant Quil-A Saponin has been demonstrated in homologous challenges and the major protective exoantigens were identified (9). The use of culture-derived exoantigens appears to be the strategy most compatible with the definition of an efficient anti-*Babesia* vaccine given by Mahoney et al. (15): immunogens are stable for more than 1 year, they can be prepared in large quantities, they prevent the establishment of clinical disease, they induce long-term protective immunity, and they do not present the restricting features of other types of babesial vaccines (4, 9a, 30).

Studies on isolates of *Babesia bovis* and *Babesia bigemina* have shown that most antigens are conserved among isolates from different geographic areas and that antigenic similarities between different *Babesia* species could be demonstrated (6, 12, 17, 18, 28). There is little information about the antigenic diversity of *B. divergens* (20, 24), which is a particularly important consideration in the development of a culture-derived *B. divergens* exoantigen-containing vaccine, which is expected to induce cross-protection against isolates from different geographic areas.

In this work, we report the identification of *B. divergens* proteins sharing common epitopes between isolates by using immunoprecipitation with various immune sera from different hosts (human, bovine, and gerbil hosts) and we have demonstrated that the exoantigens from a given isolate (*B. divergens* Rouen 1987) could induce significant protection against heterologous *B. divergens* isolates from different geographic areas.

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MATERIALS AND METHODS

***B. divergens* isolates.** The *B. divergens* isolate Rouen 1987 was obtained from a naturally infected human and maintained either in gerbils (*Meriones unguiculatus*) (CERJ, Le Genest-Saint-Isle, France) by syringe passage twice weekly (13) or in long-term in vitro culture (8).

Three bovine *B. divergens* isolates, 7107b from France (a gift from the Ecole Nationale Vétérinaire de Nantes); Munich 87 from Germany and Weybridge 8843 from the United Kingdom (Rhône-Mérieux S.A., Lyon, France) were maintained in gerbils first. Parasitized gerbil erythrocytes from each isolate were used to initiate a long-term in vitro culture in human erythrocytes.

***B. divergens* antisera.** Human antisera were obtained from splenectomized patients after recovery from acute *B. divergens* babesiosis (the Rouen 1987 isolate or the Le Mans 1988 isolate) (7) or moderate *B. divergens* babesiosis (the Marseille 1981 isolate, a gift from H. Dumon, Marseille, France) (21). Control sera were obtained from healthy donors.

Bovine antiserum against *B. divergens* Rouen 1987 was prepared as previously described (8, 9). Bovine antisera against the Y5 isolate from Ireland and the Weybridge 8843 isolate were obtained from Rhône-Mérieux. Preimmune sera from the same calves were used as controls.

Fifteen gerbil antiserum samples of each isolate were collected from gerbils that had survived an infection induced

by the inoculation of 10^4 infected erythrocytes. For each isolate, all these immune sera were pooled in order to obtain standardized gerbil immune sera. Control sera were obtained from healthy gerbils.

Protein phase separation. The proteins of *B. divergens* were phase separated in Triton X-114 (TX-114; Boehringer Mannheim GmbH, Mannheim, Germany) by using the method of Bordier (2) modified as follows. The thawed lysate (150 μl) of ^{35}S-radiolabelled parasitized erythrocytes (30% parasitemia) was incubated on ice for 1 h in 2.25 ml of a detergent solution (0.5% TX-114–PBS, which contains 0.5% TX-114 and phosphate-buffered saline [PBS; 0.15 M NaCl, 5 mM Na_2HPO_4, pH 7.2]). During incubation, samples were stirred every 10 min. After incubation, samples were centrifuged at 10,000 × *g* for 15 min at 4°C. The TX-114-insoluble pellet was washed twice by centrifugation (10,000 × *g*, 15 min, 4°C) in 0.5% TX-114–PBS and subsequently stored at −80°C. The supernatant was centrifuged again at 10,000 × *g* (15 min, 4°C) and then carefully layered over a 6% sucrose cushion in 0.06% TX-114–PBS. After incubation at 37°C for 5 min, the cloudy solution was centrifuged at 1,500 × *g* (3 min) at room temperature. Two fractions were collected: the top aqueous phase and the bottom detergent phase. The bottom detergent phase was mixed with 750 μl of PBS and stored at −80°C. The top aqueous phase was mixed with an equal volume of 11.4% TX-114–PBS, incubated for 5 min at 37°C, and centrifuged at 1,500 × *g* (3 min) at room temperature. The detergent phase was discarded, and the aqueous phase was incubated again with 11.4% TX-114–PBS. After centrifugation, the detergent phase was discarded and the aqueous phase was stored at −80°C.

Immunological methods. The indirect immunofluorescence test, [^{35}S]methionine radiolabelling of *B. divergens* in vitro culture, and immunoprecipitation assays were performed as previously described (9).

Vaccine preparation and immunization procedure. *B. divergens* Rouen 1987 culture supernatants were harvested when parasitemia reached 30 to 40% and processed as previously described in order to obtain a 7.5-fold-concentrated supernatant (9). The adjuvant Quil-A Saponin (Superfos Biosector a/s, Vedbaek, Denmark) was added to the concentrated supernatant at a final concentration of 0.5 mg/ml. The vaccine dose had a final volume of 400 μl, equivalent to 1.5 ml of parasitized culture supernatant. The supernatant of normal erythrocytes cultured in the same conditions in vitro was processed as described above.

Ten days before the vaccine trial, 90 gerbils were randomly distributed into 18 cages of 5 animals each, i.e., 9 groups of 10 animals. On days 0 and 21, the vaccination groups Av, Bv, Cv, Dv, and Ev were injected subcutaneously with a vaccine dose while the control groups Ac, Bc, Cc, and Dc received a placebo dose. For the immunofluorescence assays, blood specimens were collected from five gerbils of group Ev by puncturing the heart after lethal anesthesia on day 21 or 42.

Challenge procedure. On day 63, all remaining gerbils were inoculated intraperitoneally with 1.6×10^6 parasitized gerbil erythrocytes resuspended in 0.1 ml of RPMI 1640 (Gibco BRL, Paisley, Scotland). Animals in the various groups were injected with the different *B. divergens* isolates as follows: group A, isolate Rouen 1987; group B, isolate 7107b; group C, isolate Weybridge 8843; and group D, isolate Munich 87. After challenge, the gerbils were not handled until day 80, while dead animals were counted daily and removed from the cage. Blood specimens for immunofluorescence were obtained after lethal anesthesia from all surviving gerbils on day 80.

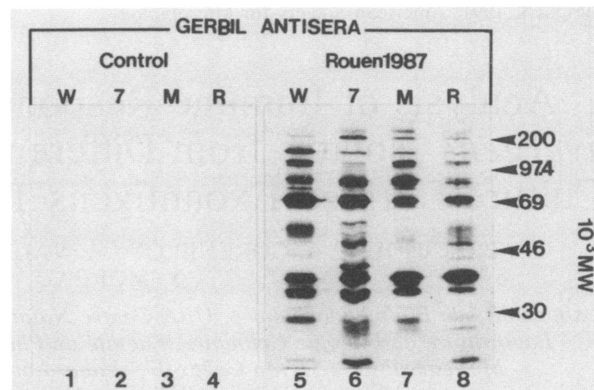

FIG. 1. Immunoprecipitations of [^{35}S]methionine-radiolabelled *B. divergens* isolates by gerbil preimmune sera (lanes 1 to 4) or gerbil antisera raised against the *B. divergens* Rouen 1987 isolate (lanes 5 to 8). Isolate abbreviations: W, Weybridge 8843; 7, 7107b; M, Munich 87; and R, Rouen 1987.

Statistical analysis. The reciprocal survival time in vaccinated and control gerbils was the variable analyzed by statistical procedures. Differences were compared by crossed-factor variance analysis; the first factor was the nature of the *B. divergens* isolate (Rouen 1987, 7107b, Weybridge 8843, Munich 87), and the second was the nature of the treatment received (vaccine or placebo).

RESULTS

Identification of major immunogenic polypeptides in different *B. divergens* isolates. Immunoprecipitations with pooled gerbil sera raised against the Rouen 1987 isolate were performed by using [^{35}S]methionine-radiolabelled *B. divergens* total antigens of four isolates: Weybridge 8843 (Fig. 1, lane 5), 7107b (lane 6), Munich 87 (lane 7), and Rouen 1987 (lane 8). For all four isolates, the major immunogenic parasitic antigens recognized by the gerbil antisera were polypeptides of 220, 200, 150, 110 and 120 (doublet), 85 and 87 (doublet), 78, 70, 37, 35, 29, and 26 kDa. Slight differences were, however, observed between the different samples (Table 1). For instance, the 55- and 40-kDa antigens were observed only in 7107b, the 50- and 17-kDa antigens were absent in Munich 87, and the 48-kDa antigens was absent in both Weybridge 8843 and Munich 87. Immunoprecipitation controls were negative (Fig. 1, lanes 1 to 4).

Identification of cross-reactive epitopes between *B. divergens* isolates. To identify cross-antigenicity between different *B. divergens* isolates, different pooled isolate-specific sera were tested on radiolabelled proteins of heterologous isolates (Fig. 2 to 4). The cross-antigenicity through the humoral response was tested on three potential *B. divergens* hosts, gerbils (Fig. 2), oxen (Fig. 3), and humans (Fig. 4). Pooled immune sera obtained from gerbils experimentally infected with Weybridge 8843 (Fig. 2, lane 5 to 8), 7107b (lanes 9 to 12), or Munich 87 (lanes 13 to 16) were used to immunoprecipitate the homologous isolates and the three [^{35}S]methionine-radiolabelled heterologous isolates. Immunodominant parasitic antigens immunoprecipitated by the three heterologous gerbil antisera were similar (corresponding to 220, 200, 150, 110 and 120 doublet, 87, 70, 50, 37, 35, 29, and 26 kDa) but again showed slight differences from one

TABLE 1. Antigens of various *B. divergens* isolates from different geographic areas immunoprecipitated by antisera raised against the *B. divergens* Rouen 1987 isolate

Antigen (kDa)	[35S]methionine-radiolabelled isolate			
	Rouen 1987	Weybridge 8843	Munich 87	7107B
220	+	+	+	+
200	+	+	+	+
150	+	+	+	+
110 and 120	+	+	+	+
85 and 87	+	+	+	+
78	+	+	+	+
70	+	+	+	+
55	−	−	−	+
50	+	+	−	+
48	+	−	−	+
40	−	−	−	+
37	+	+	+	+
35	+	+	+	+
29	+	+	+	+
26	+	+	+	+
17	+	+	−	+

isolate to another: the 40- and 48-kDa antigens were detected only with sera raised against Weybridge 8843 and 7107b, and the 17-kDa antigen was not precipitated with antisera against Munich 87.

Heterologous immunoprecipitations performed with bovine sera obtained after experimental infection with the Rouen 1987 (Fig. 3, lanes 5 to 8), Weybridge 8843 (lanes 9 to 12), or Y5 (lanes 13 to 16) isolate exhibited, as with gerbil antisera, a strong cross-reaction against common polypeptides from the different isolates. Antisera from human patients who had recovered from acute or moderate *B. divergens* babesiosis were also tested against three French isolates, Rouen 1987 (Fig. 4, lanes 5 to 8), Le Mans 1988 (lanes 9 to 12), and Marseille 1981 (lanes 13 to 16). As in the natural bovine host or *B. divergens* babesiosis experimental model, the human humoral response against immunodominant parasitic antigens was also constant. As was true for the bovine or gerbil antisera, the humoral response of humans (an unusual host) displayed some minor variations.

TX-114-soluble *B. divergens* antigens. [^{35}S]methionine-radiolabelled *B. divergens*-infected human erythrocytes from in vitro culture were solubilized with TX-114 and submitted to a temperature-dependent phase separation with TX-114 in order to obtain the aqueous and detergent phase of the babesial antigen preparation. Immunoprecipitations on TX-114-extracted antigens were performed with four gerbil antiserum samples raised against Rouen 1987 (Fig. 5, lanes 5 and 6), Weybridge 8843 (lanes 7 and 8), 7107b (lanes 9 and 10), and Munich 87 (lanes 11 and 12), using either the aqueous (lanes 5, 7, 9, and 11) or detergent phase (lanes 6, 8, 10, and 12). By using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), a clear discrimination was observed between the antigens partitioning into the aqueous phase (Fig. 5, lane 1) and the detergent phase (lane 2). In spite of some variations, principally in the strength of the antibody-mediated immune response, the *B. divergens* Rouen 1987 antigens revealed by partitioning into the aqueous phase and immunoprecipitated by all the sera correspond to polypeptides of 150, 85 and 90 (doublet), 69 and 70 (doublet), 48, 44 and 45 (doublet), 38, 34, and 32 kDa. The immunoprecipitated detergent phase-soluble antigens were polypeptides of 85, 70, 50, and 37 kDa.

Immunization of gerbils by *B. divergens* Rouen 87 in vitro culture supernatants and heterologous challenges. The survival rate of vaccinated gerbils and placebo controls was recorded daily. Cumulative mortality rates of gerbils are summarized in Fig. 6. Mortality rates varied in placebo control groups: 90% for Ac (Rouen 1987), 80% for Bc (7107b), 100% for Cc (Weybridge 8843), and 100% for Dc (Munich 87). All the vaccinated gerbils challenged with the two French isolates (Rouen 1987 and 7107b) survived and did not exhibit any obvious symptoms. In contrast, 20 and 40% of the vaccinated gerbils died after challenge with Weybridge 8843 and Munich 87, respectively. Reciprocal survival times are shown in Table 2. A two-factor (crossed-factor) variance analysis between the four groups reveals the following observations: (i) A high protection is conferred by *B. divergens* Rouen 1987 culture supernatant against the three heterologous isolates. (ii) Differences in virulence between the various isolates can be observed (Weybridge 8843 and Munich 87 isolates are more virulent in the gerbil than the two French isolates). (iii) When gerbils are challenged with either the Weybridge or Munich isolate, the mean of their reciprocal survival times are significantly higher than when they are challenged with Rouen 1987 or 7107b isolate.

Antibodies against *B. divergens* can already be detected after the first vaccine injection with a mean titer of 1/140, and the second vaccine injection did not enhance the titer (group

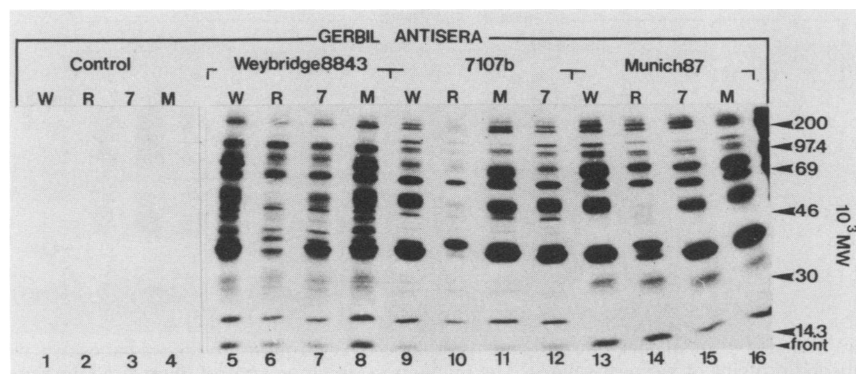

FIG. 2. Immunoprecipitations of [^{35}S]methionine-radiolabelled *B. divergens* isolates by control gerbil antisera (lanes 1 to 4) or by gerbil antisera raised against the Weybridge 8843 isolate (lanes 5 to 8), the 7107b isolate (lanes 9 to 12), or the Munich 87 isolate (lanes 13 to 16). Isolate abbreviations: W, Weybridge 8843; R, Rouen 1987; 7, 7107b; and M, Munich 87.

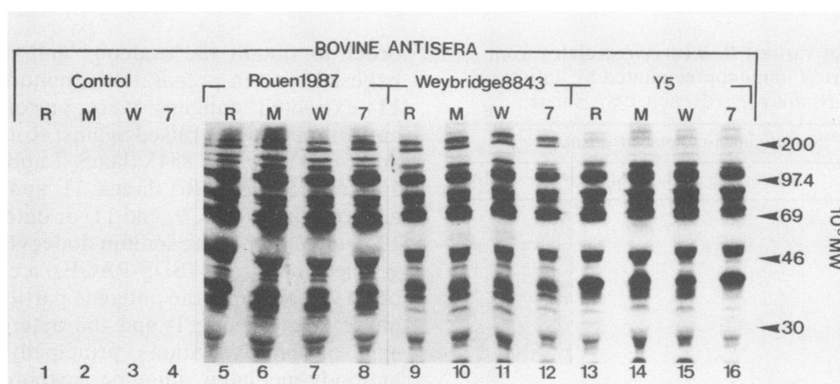

FIG. 3. Parasitic proteins from different *B. divergens* isolates were immunoprecipitated by different bovine antisera after [^{35}S]methionine radiolabelling. No babesial antigens were immunoprecipitated by control serum (lanes 1 to 4). Whatever the bovine serum, whether it was specific for the Rouen 1987 isolate (lanes 5 to 8), the Weybridge 8843 isolate (lanes 9 to 12) or the Y5 isolate (lanes 13 to 16), the immunoprecipitated antigens of the isolates were similar. Isolate abbreviations: R, Rouen 1987; M, Munich 87; W, Weybridge 8843; 7, 7107b.

E [data not shown]). After the challenge procedure, a dramatic increase in the mean antibody titer of the sera of the surviving gerbils was observed in the vaccinated animals and the rare controls that survived challenge. The mean antibody titers observed at day 80 were 1/1,950 for the gerbils challenged with *B. divergens* Rouen 87 isolate (homologous to the vaccine isolate), 1/2,900 for 7107b isolate, 1/7,250 for Munich 87 isolate, and 1/9,100 for Weybridge 8843 isolate (Table 3).

DISCUSSION

Despite economic losses in European countries due to the bovine hemoprotozoan *B. divergens*, no effective immunoprophylaxis for this bovine babesiosis has yet been developed. Recently, it has been demonstrated that immunization of the laboratory rodent *Meriones unguiculatus* (Mongolian gerbil) with concentrated supernatants from in vitro cultures of *B. divergens* induces protection against virulent homologous challenge and that the host humoral response was conserved against major babesial antigens (9). Demonstration of exoantigen-induced heterologous protection and comparative analyses of the immunogenic *B. divergens* polypeptides among different isolates are needed to ensure that a subunit vaccine will protect cattle against isolates from different geographic areas.

B. divergens antigens inducing an antibody-mediated immune response were compared at one and the same time in different hosts and in heterologous immunoprotections. The occurrence of numerous epitopes common to various *B. divergens* isolates (Rouen 1987, 7107b, Weybridge 8843, and Munich 87) was detected with hyperimmune sera of three different hosts. This antigenic conservation among different isolates was confirmed by immunoprecipitations performed on [^{35}S]methionine-radiolabeled babesial proteins solubilized in TX-114. Following a temperature-induced phase separation, hydrophilic proteins were partitioned into the aqueous phase whereas amphiphilic proteins were recovered in the detergent phase. The fact that all the anti-*B. divergens* sera did recognize parasitic antigens with similar physicochemical characteristics confirmed that all the isolates tested had similar antigens. These results are in agreement with results previously described for *B. bovis* and *B. bigemina* isolates (6, 11, 14, 16, 22). Similarly, cross-antigenicity and cross-protection between different *Babesia* species is well documented: for example, *B. bovis* and *B. bigemina* (19, 23, 29), *B. rhodaini* and *B. microti* (3), and *B. bigemina* and *B. major* (31). This extensive cross-immunogenicity suggests the conservation of several epitopes between babesial antigens from different isolates. These cross-reactive epitopes probably possess a functional importance which could be related to a immunoprotective function; on the other hand, they may serve to delay the recognition of the protective antigen(s) by humoral or other immune mechanisms as has

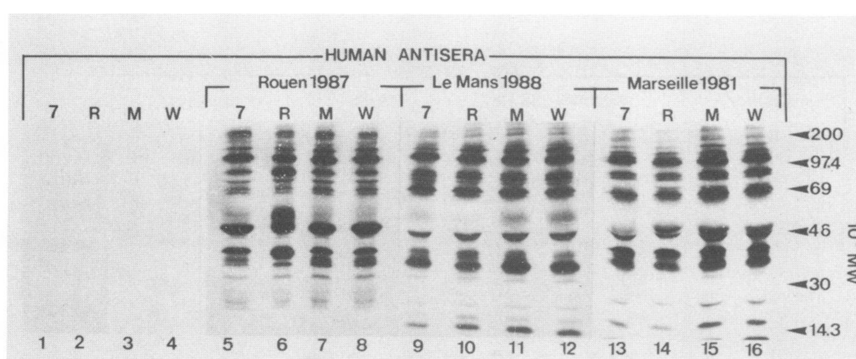

FIG. 4. Human antisera obtained from splenectomized patients after recovery from *B. divergens* babesiosis were used to immunoprecipitate *B. divergens* radiolabelled proteins of various isolates. The immunoprecipitation control (human nonimmune serum) is shown in lanes 1 to 4. The Rouen 1987 (lanes 5 to 8) and Le Mans 1988 (lanes 9 to 12) human antisera were collected after acute *B. divergens* babesiosis. The Marseille 1981 human antiserum (lanes 13 to 16) was obtained after moderate *B. divergens* babesiosis. Isolate abbreviations: 7, 7107b; R, Rouen 1987; M, Munich 1987; W, Weybridge 8843.

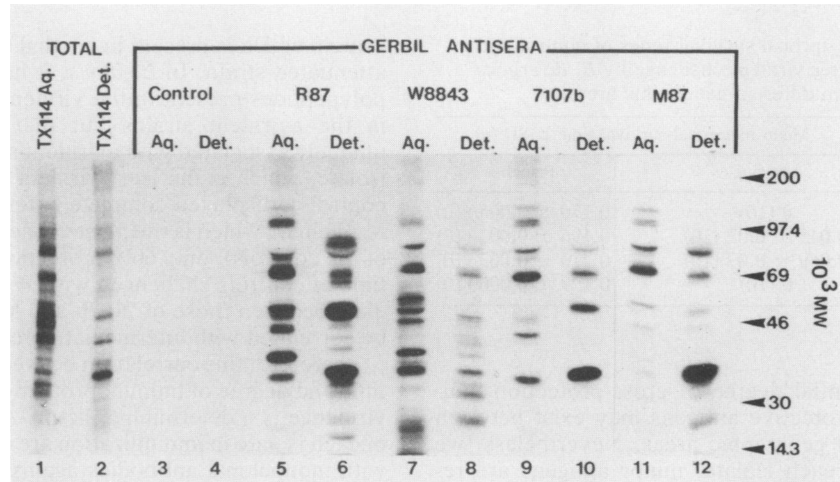

FIG. 5. The [^{35}S]methionine-radiolabelled in vitro cultures of the *B. divergens* Rouen 1987 isolate were solubilized in TX-114, and following a temperature-induced phase separation, the hydrophilic antigens were recovered in the aqueous phase and the amphiphilic antigens were recovered in the detergent phase. After SDS-PAGE was performed, the hydrophilic and amphiphilic proteins are shown in lane 1 (TX114 Aq.) and 2 (TX114 Det.), respectively. Different gerbil immune sera raised against the Rouen 1987 isolate (lanes 5 and 6), the Weybridge 8843 isolate (lanes 7 and 8), the 7107b isolate (lanes 9 and 10), or the Munich 87 isolate (lanes 11 and 12) were used to immunoprecipitate either the aqueous-phase proteins (Aq.) or the detergent-phase proteins (Det.). With the control nonimmune sera, no babesial antigens were immunoprecipitated in the aqueous or detergent phase (lanes 3 and 4).

been supposed for other parasites, such as *Plasmodium falciparum* (1) or *Trypanosoma brucei* (5).

A vaccination protocol was designed to assess the protection value of culture-derived soluble antigens of *B. divergens* Rouen 1987 to induce a heterologous immunity against isolates from different geographic areas. Gerbils were fully protected against challenge with the homologous Rouen 1987 isolate and the heterologous 7107b isolate and partially protected against the heterologous Weybridge (80% protection) and Munich (60% protection) isolates. At least three parameters could be implicated in the differences observed in heterologous protection: antigenic status of each isolate, virulence, and geographic origin. The antigenic status and virulence are probably the most important biological parameters in heterologous protection against *B. divergens* as observed in various *B. bovis* strains (11, 27) and *P. falciparum* (1). The importance of the geographic origin of the isolates until now has been difficult to establish because (i) it could represent a confounding variable that is not causal by itself but is closely associated with virulence or antigenic structure and (ii) the genetic recombination observed in natural populations of parasitic protozoa increases the complexity in the characterization of individual natural strains as suggested by the hypothesis of clonal reproduction for parasitic protozoa (26). The antigenic status was found to be conserved among different isolates of *B. divergens* and the

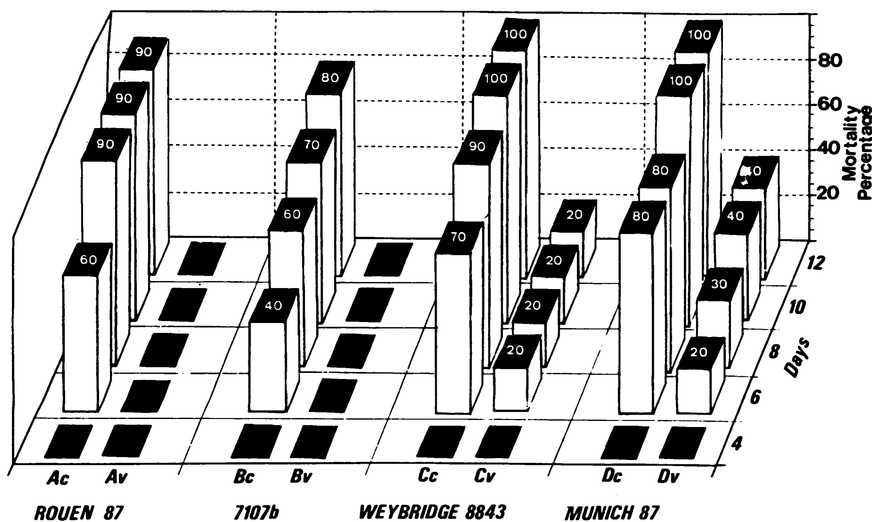

FIG. 6. Mortality percentages of heterologously challenged gerbils determined on days 4, 6, 8, 10, and 12. The gerbils of groups Ac, Bc, Cc, and Dc were vaccinated with *B. divergens* Rouen 1987 (in vitro culture supernatant). The gerbils of groups Av, Bv, Cv, and Dv were injected with placebo. Animals of groups Ac and Av were homologously challenged with the Rouen 1987 isolate. All the other groups were heterologously challenged with the 7107b isolate (groups Bc and Bv), the Weybridge 8843 isolate (groups Cc and Cv), or the Munich 87 isolate (groups Dc and Dv).

TABLE 2. Mean reciprocal survival times of control and vaccinated gerbils after virulent challenge by *B. divergens* isolates from different geographic areas

Challenge isolate	Mean reciprocal survival time ± SD (*n*)	
	Vaccine	Placebo
Rouen 1987	0 (10)	0.170 ± 0.005 (10)
Weybridge 8843	0.045 ± 0.02 (10)	0.196 ± 0.002 (10)
Munich 87	0.069 ± 0.09 (10)	0.210 ± 0.003 (10)
7107B	0 (10)	0.129 ± 0.006 (10)

existence of a substantial degree of cross-protection indicates that common protective antigens may exist between isolates from different geographic areas. Nevertheless, we can suppose that, in each isolate, minor antigens are required to provide complete heterologous protection. We have compared the antigens immunoprecipitated with the sera of immune gerbils against the vaccinal Rouen 1987 isolate. This comparison has revealed the following observations. (i) In the 7107b isolate (100% protection), all the immunoprecipitated antigens were found in the Rouen 1987 isolate. (ii) In the Weybridge 8843 isolate (80% protection), the 55-, 48-, and 40-kDa antigens were not immunoprecipitated. (iii) In the Munich 87 isolate (60% protection), the 55-, 50-, 48-, 40-, and 17-kDa antigens were not immunoprecipitated. As the immunoprecipitation tests were performed with pooled immune gerbil antisera and as we have demonstrated that the variability among individuals was very low (8, 9), it can be assumed that the variations in number and intensity of the immunoprecipitated bands have biological significance. Other data such as the strong inhibition of the in vitro development of *B. divergens* Rouen 1987 (50% inhibitory concentration of 20 μg/ml) by a monoclonal antibody against a 17-kDa antigen (submitted for publication) and the complete protection in homologous challenge, of gerbils immunized with a gel filtration-defined fraction (50, 40, 37, and 17 kDa) for the *B. divergens* Rouen 1987 exoantigens (unpublished results) indicated that incomplete protection against Weybridge or Munich isolate could be partly linked to some absence of response to these antigens. Nevertheless, variations in heterologous protection could also be due to an association of the antigenic status and virulence of the challenge isolates. A variable virulence of the isolates was demonstrated by the statistical analysis of the mean reciprocal survival times of control animals after challenge with the different isolates: the Weybridge 8843 and Munich 87 isolates were significantly more virulent and killed their hosts more rapidly than the others. The molecular basis for virulence is not yet known in *B. divergens* but in *B. microti*, Tetzlaff et al. (25) have characterized a gene associated with virulence. This gene encodes a *B. microti*-specific 54-kDa protein which is present in a lethal strain and absent from an attenuated strain. In *B. bovis*, it has been shown that most polypeptides present in the virulent strains are also present in the avirulent strains but that some polypeptides are uniquely associated with avirulent strains (11, 27). The 7107b isolate, which is the least virulent (longest survival time of controls), displayed complete heterologous protection; with Munich 87, which is the most virulent (shortest survival time of the controls), only 60% protection was seen. The survival time of controls challenged with Weybridge 8843 is intermediate between those of 7107b and Munich 87 and appears to be correlated with intermediate protection (80% protection).

TABLE 3. Antibody titers ($\log_{10}$) observed at day 80 (17 days after heterologous challenge) in gerbils immunized with *B. divergens* Rouen 1987 (in vitro culture supernatant)

Challenge isolate	Antibody titer, mean ± SD (*n*)	
	Vaccine	Placebo
Rouen 1987	3.29 ± 0.11 (10)	3.10 ± 3.10 (1)
Weybridge 8843	3.86 ± 0.14 (8)	
Munich 87	3.96 ± 0.05 (6)	
7107B	3.46 ± 0.04 (10)	2.35 ± 0.15 (2)

Therefore, this correlation between the reciprocal survival time and degree of immunoprotection could indicate that the virulence is a determining factor. Assays using exoantigens of each isolate in immunization are under way; future studies with monoclonal antibodies against these 50-, 40-, 37-, and 17-kDa *B. divergens* antigens are needed to precisely document the impact of antigen variation and virulence in cross-protection against *B. divergens* heterologous challenges in gerbils.

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